

Identification of polymorphisms in the EPCR gene associated with
thrombotic risk

5 The present invention relates to methods for assessing a genetic risk of thrombosis.

The protein C (PC) system is an important natural anticoagulant mechanism. PC, a vitamin K-dependent zymogen, is activated at the endothelial surface when thrombin binds to thrombomodulin, a protein that transforms the procoagulant enzyme into a potent activator of PC. In the
10 presence of its cofactor, protein S, activated protein C (aPC) inactivates factor Va and VIIIa, thereby reducing thrombin generation (Dahlbäck et al., (1995)).

Another factor contributing to protein C activation - endothelial cell activated protein C receptor (EPCR) - was discovered more recently at the surface of endothelial cells (Fukudome et al. (1994)). This receptor, which can
15 bind PC or aPC with the same affinity ($K_d = 30$ nM), is mainly expressed on endothelial cells of large vessels (Laszik et al. (1997) ; Ye et al. (1999) ; Fukudome et al. (1998)).

Functional studies performed *in vitro* showed a 3- to 5-fold increase in the PC activation rate by the membrane thrombin-thrombomodulin complex when
20 PC is bound to its receptor (Stearns-Kurosawa et al. (1996)). This increase results from a significant effect of EPCR on the K_m for PC activation by the thrombin-thrombomodulin complex. Indeed, without EPCR intervention, this K_m is significantly higher (1 μ M) than the circulating concentration of PC (60-70 nM). By presenting PC to the thrombin-thrombomodulin complex, and owing to
25 its lateral mobility, EPCR reduces the K_m and thereby allows the interaction to occur.

In view of these functions, EPCR was expected to intervene in the physiological regulation of coagulation. Evidence for an important role of this type came from baboon studies (Taylor et al. (2000) ; Taylor et al. (2001)), in
30 which an 88% reduction in aPC generation induced by thrombin infusion was observed in animals that had been pretreated with anti-EPCR antibodies blocking the PC/EPCR interaction.

EPCR is a 46-kD type 1 transmembrane glycoprotein homologous to major histocompatibility complex class I/CD1 family proteins (Fukudome et al. (1994) ; Villoutreix et al. (1999) ; Liaw et al. (2001). This 221-amino-acid (aa) protein comprises an extracellular domain, a 25-aa transmembrane domain, and a short (3 aa) intracytoplasmic sequence. The gene is located on chromosome 20 (Hayashi et al. (1999), at position q11.2; it spans 8 kb and comprises 4 exons (Hayashi et al. (1999) ; Simmonds et al. (1999). The first exon encodes the 5' untranslated region and the signal peptide, exons 2 and 3 most of the extracellular domain, and exon 4 the remaining parts of the protein and the 3' untranslated region. The proximal part of the promoter was recently functionally characterized (Rance et al. (2003).

Several authors have reported the presence in plasma of a soluble form of EPCR (sEPCR) (Kurosawa et al. (1997) ; Kurosawa et al. (1998)) that probably lacks the transmembrane domain and cytoplasmic tail. This sEPCR is detected as a single species of 43 kD, resulting from shedding of membrane EPCR by the action of a metalloprotease (Xu et al. (2000)) which is stimulated by thrombin and by some inflammatory mediators (Gu et al. (2000)). Soluble EPCR binds PC and aPC with similar affinity (Fukudome et al. (1996) ; Regan et al. (1996)), but its binding to aPC inhibits the anticoagulant activity of aPC by blocking its binding to phospholipids and by abrogating its ability to inactivate factor Va (Liaw et al. (2000)). By contrast with the membrane-associated form of EPCR, PC binding to sEPCR does not result in enhanced aPC generation by the thrombin-thrombomodulin complex (Regan et al. (1996)). A recombinant sEPCR has recently been crystallized (Oganesyan et al., 2002).

Dysfunctional EPCR-dependent activation of PC would potentially be thrombogenic. A loss of function could result from mutations leading to decreased expression of membrane EPCR. A 23-bp insertion has been reported to impair EPCR functions by leading to the synthesis of a truncated protein that is not expressed on endothelial surfaces (Biguzzi et al. (2001)). Although initially identified in thrombophilic subjects (von Depka et al. (2001)), the role of this mutation in thrombosis is difficult to assess because its allelic frequency is low (von Depka et al. (2001) ; Akar (2002) ; Poort et al. (2002) ; Galligan et al. (2002)). Point mutations were recently described within the

promoter region (Biguzzi et al. (2002)) of the gene in four thrombophilic subjects, but the involvement of these mutations in gene regulation could not be clearly demonstrated.

Another possible mechanism leading to dysfunction of the EPCR-mediated coagulation-regulating mechanism consists of mutations (or polymorphisms) leading to increased levels of sEPCR. Indeed, increased sEPCR levels may be prothrombotic, as sEPCR can inhibit aPC activity, as well as PC activation, by competing for PC with membrane-associated EPCR.

Two recent studies show that sEPCR levels vary widely among healthy subjects (Stearns-Kurosawa et al. (2002) ; Stearns-Kurosawa et al. (2003). While sEPCR levels are between 75 and 178 ng/mL in 80% of subjects, the remaining 20% of subjects have values between 200 and 700 ng/mL. This bimodal distribution has repeatedly been reported in both French and Italian populations (Stearns-Kurosawa et al. (2003)).

Several polymorphisms in the EPCR have been studied for possible association with the risk of thrombosis.

G/A polymorphism affecting nt 6936 has been described in two different studies, both of which showed a very similar frequency of the G allele in thrombophilic and control Caucasian subjects (Espana et al 2001, wherein the polymorphism was designated A7685 ; Poort et al 2002, wherein the polymorphism was designated A4300G).

The 7014 G/C polymorphism has also been reported in three studies (Espana et al 2001, Galligan et al 2002 and Medina et al 2003) wherein the polymorphism was designated G7763C, G5252C and G4678C, respectively.

Finally, a third polymorphism corresponding to nt 4868 has been described by Poort et al (2002) with T3997C numbering.

In the XVIIIth Congress of the International Society on Thrombosis and Haemostasis (July 2001), Espana et al reported that an EPCR allele bearing the 4678 C polymorphism (that corresponds to nt 7014 polymorphism described here) has a protective effect against thrombosis, and at the XIX Congress of the International Society on Thrombosis and Haemostasis (July 2003), Navarro et al described a similar effect of this allele in thrombophilic carriers of the factor V Leiden mutation.

The inventors now extensively analysed the EPCR gene and identified several polymorphisms that were in complete linkage disequilibrium, defining three haplotypes (designated A1, A2 and A3). One of three haplotypes was associated with increased sEPCR levels, offering the first evidence that inter-individual variations in sEPCR levels are genetically regulated.

As sEPCR can inhibit both aPC generation and aPC activity, the inventors examined whether the haplotype associated with high sEPCR levels carried an increased risk of venous thrombosis. On comparing 338 subjects with thrombosis and 338 age- and sex-matched healthy controls, the inventors observed a significantly higher allelic frequency of this haplotype in the cases.

Based on these results, the present invention provides a method for distinguishing A1, A2 and A3 haplotypes in the EPCR receptor, the latter being associated with a higher risk to develop thrombosis.

This method is thus useful to determine the risk of developing thrombosis, especially venous thrombosis, in a subject.

EPCR receptor

As above mentioned, the human EPCR receptor has been cloned. EPCR has also been characterized in other species, such as mouse and bovine :

	Gene (EMBL/Genbank)	mRNA (EMBL/Genbank)	Protein	
			Swissprot	PIR
Human	AF106202	L35545	Q9UNN8	A55365
Mice	AF162695 AF224271	L39017	Q64695	A55945
Bovine		L39065	Q28105	B55945

In the context of the present invention, the term "EPCR" or "EPCR receptor" refers to the EPCR receptor of any species, especially in human, but also in other mammals or vertebrates to which the methods of the invention can

apply, if desired. The term "*subject*" thus refers to any human patient or any mammal or vertebrate.

The human EPCR gene sequence is available on Genbank (access number : AF106202), and is also shown in SEQ ID NO:1 (A2 haplotype). In this
5 sequence, the starting codon for the open reading frame is located at nucleotide 2337.

A chromosomal sequence is also available on Genbank (AL 356652), and corresponds to the A1 haplotype. In that sequence, the EPCR gene is located from nucleotide 29655 to nucleotide 37818.

10 A sequence of the A3 haplotype is represented as SEQ ID No: 2.

Haplotypes

The inventors found various nucleotide polymorphisms (SNPs) in the EPCR gene. In the present invention, these polymorphisms are sometimes
15 referred to as "the polymorphic positions of interest".

To make it simple, these polymorphisms are herein numbered according to SEQ ID No:1, starting from nucleotide 1.

However it should be noted that this numbering is arbitrary.

The identified SNPs defined three haplotypes, that were designated A1,
20 A2 and A3.

As shown in figure 3, A1 and A2 were major haplotypes, with allelic frequencies of 0.48 and 0.45, respectively. The A1 haplotype consisted of a combination of T at nt 3787, A at nt 3877, C at nt 4216, C at nt 4868, A at nt 5233, C at nt 5760, C at nt 6333, C at nt 7014, G at nt 7968 and A at nt 7999.
25 The A2 haplotype was a combination of C at nt 3787, G at nt 3877, G at nt 4216, T at nt 4868, G at nt 5233, T at nt 5760, T at nt 6333, G at nt 7014, A at nt 7968 and G at nt 7999. A3, the least common haplotype (allelic frequency 0.07), differs from A2 haplotype at four nucleotide positions (G at nt 1651, C at nt 3610, A at nt 4216 and G at nt 6936).

30 The A3 haplotype is thus defined as the simultaneous presence of the following polymorphisms :

- G at position 1651
- C at position 3610

- A at position 4216
- G at position 6936
- C at position 3787
- G at position 3877
- 5 - T at position 4868
- G at position 5233
- T at position 5760
- T at position 6333
- G at position 7014
- 10 - A at position 7968
- G at position 7999

The isolated nucleic acid of the EPCR receptor gene with the A3 haplotype is also part of the present invention.

- 15 A subject of the invention is thus an isolated nucleic acid encoding the EPCR receptor, which nucleic acid comprises the EPCR gene sequence with the simultaneous presence of :

- G at position 1651
- C at position 3610
- 20 - A at position 4216
- G at position 6936
- C at position 3787
- G at position 3877
- T at position 4868
- 25 - G at position 5233
- T at position 5760
- T at position 6333
- G at position 7014
- A at position 7968
- 30 - G at position 7999

Such nucleic acid preferably comprises SEQ ID No: 2.

The identification of any of :

- G at position 1651

- C at position 3610
- A at position 4216
- G at position 6936

is sufficient to identify the A3 haplotype.

- 5 Conversely, the presence of a C nucleotide at position 4216 marks the existence of the A1 haplotype, whereas the presence of a G nucleotide at position 4216 marks the existence of the A2 haplotype.

- 10 It is therefore provided a method for genotyping the EPCR gene, which method comprises identifying the polymorphism of the EPCR gene at position 4216 (SEQ ID No: 1), wherein the presence of C at position 4216 shows the A1 haplotype. The presence of G at position 4216 shows the A2 haplotype, and the presence of A shows the A3 haplotype, the latter being associated with a higher risk to develop thrombosis. It was further shown that the individuals carrying the A1 haplotype have a lower thrombotic risk (Medina et al., 2004).

- 15 Various methods for identifying the polymorphisms at position 4216 are described in greater details below, in connection with the identification of the A3 allele. The skilled person will recognize that the same type of methods may be used with regard to the identification of the A1 or A2 haplotypes.

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Thrombosis risk

- 25 The present invention provides an *in vitro* method for determining the risk of developing thrombosis in a subject, which method comprises identifying polymorphisms of EPCR gene on at least one of positions 1651, 3610, 4216, and 6936, wherein the presence of G at position 1651, C at position 3610, A at position 4216, or G at position 6936 is indicative of a higher risk to develop thrombosis in comparison with a control subject that does not show the same polymorphisms.

- 30 In a preferred embodiment, the method comprises identifying polymorphisms of EPCR gene at positions 1651, 3610, 4216, 6936, 3787, 3877, 4868, 5233, 5760, 6333, 7014, 7968, 7999 of SEQ ID No: 1, wherein the simultaneous presence of :

- G at position 1651
- C at position 3610

- A at position 4216
- G at position 6936
- C at position 3787
- G at position 3877
- 5 - T at position 4868
- G at position 5233
- T at position 5760
- T at position 6333
- G at position 7014
- 10 - A at position 7968
- G at position 7999

are designated A3 haplotype and, when present on at least one allele, are indicative of a higher risk to develop thrombosis in comparison with a control subject without any A3 allele.

- 15 A higher risk to develop thrombosis means a significantly greater risk in carriers of the A3 allele than in non carriers.

- Such a relative risk is frequently estimated through case-control studies and the use of the odds ratio (OR). An OR of 1.1 corresponds to an increase of 10% of the risk of developing disease and an OR of 2 corresponds to a 100 %
20 increased risk.

The risk of developing thrombosis is connected with increased plasma levels of soluble EPCR.

In the context of the present invention, the term "thrombosis" means formation of a clot in a blood vessel or in a heart cavity.

- 25 In a preferred embodiment, venous thrombosis is encompassed.

Consequently, the identification of the A3 haplotype of the EPCR receptor in a subject is further indicative of a higher risk to develop thromboembolic diseases, *e.g.* pulmonary embolism or deep venous thrombosis (DVT).

- 30 For instance, knowing the genetic status of an individual with respect to the EPCR haplotype is particularly useful to prevent recurrence of lung embolisms, that are fatal in 20 % of cases.

Within the meaning of the invention, "thrombosis" as above defined encompasses other thrombotic states such as arterial thrombosis as well as thrombotic microangiopathy or intravascular disseminated coagulation.

5 ***Identification of the presence of the A3 allele***

The methods described above for determining the thrombosis risk involve the identification of the A1, A2 or A3 haplotype of the EPCR gene.

Such identification may be performed by any technique well known from the person skilled in the art.

10 In practicing the methods of the invention, an individual's polymorphic pattern with regard to the haplotype of interest can be established by obtaining DNA from the individual and determining the sequence at the polymorphic positions of the EPCR receptor gene interest.

15 The DNA may be obtained from any cell source. Non-limiting examples of cell sources available in clinical practice include blood cells, buccal cells, cervicovaginal cells, epithelial cells from urine, fetal cells, hair, or any cells present in tissue obtained by biopsy, e.g. tumor biopsy. Cells may also be obtained from body fluids, including without limitation blood, saliva, sweat, urine, cerebrospinal fluid, feces, and tissue exudates at the site of infection or
20 inflammation. DNA may be extracted from the cell source or body fluid using any of the numerous methods that are standard in the art. It will be understood that the particular method used to extract DNA will depend on the nature of the source. In another embodiment, the analysis is achieved without extraction of the DNA, e.g. on whole blood (Mercier et al, 1990).

25 Determination of the sequence of the DNA at the polymorphic positions of the A1, A2 and A3 haplotype is achieved by any means known in the art. Numerous strategies for genotype analysis are available (Antonarakis et al., 1989 ; Cooper et al., 1991 ; Grompe, 1993). The strategies include, but are not limited to, direct sequencing, restriction fragment length polymorphism (RFLP)
30 analysis, hybridization with allele-specific oligonucleotides, allele-specific PCR, PCR using mutagenic primers, ligase-PCR, HOT cleavage, denaturing gradient gel electrophoresis (DGGE), temperature denaturing gradient gel electrophoresis (TGGE), single-stranded conformational polymorphism (SSCP)

and denaturing high performance liquid chromatography (Kuklin et al., 1997). Direct sequencing may be accomplished by any method, including without limitation chemical sequencing, using the Maxam-Gilbert method ; by enzymatic sequencing, using the Sanger method ; mass spectrometry sequencing ; sequencing using a chip-based technology (see e.g. O'Donnell-Maloney et al., 1996); and real-time quantitative PCR. Preferably, DNA from a subject is first subjected to amplification by polymerase chain reaction (PCR) using specific amplification primers. However several other methods are available, allowing DNA to be studied independently of PCR, such as the oligonucleotide ligation assay (OLI), rolling circle amplification (RCA) or the InvaderTM assay.

In a particular embodiment of the invention, it is provided an in vitro method for identifying at least one polymorphism of an haplotype of the EPCR receptor associated with thrombosis in a subject, which method comprises analyzing genomic DNA of a biological sample, in at least one of the regions of the EPCR gene, located around positions 1651, 3610, 4216 and 6936, preferably as well as in the regions of the EPCR gene located around positions 3787, 3877, 4868, 5233, 5760, 6333, 7014, 7968 and 7999 of SEQ ID No: 1;

wherein the presence of :

- G at position 1651
- C at position 3610
- A at position 4216, or
- G at position 6936, more particularly the simultaneous presence of these SNPs in combination with the following :
- C at position 3787
- G at position 3877
- T at position 4868
- G at position 5233
- T at position 5760
- T at position 6333
- G at position 7014
- A at position 7968, and
- G at position 7999

when present on at least one allele, are indicative of a higher risk to develop thrombosis, in comparison with a control subject.

The analysis may preferably comprise a step of amplification (e.g. by PCR) of said regions of the genomic DNA.

5 In a particular embodiment the analysis is undertaken on isolated (*i.e.* extracted) genomic DNA.

A preferred technique for identifying the polymorphisms of the EPCR receptor includes sequencing or RFLP analysis.

To identify the G6936 polymorphism, one can for instance create a
10 restriction site for the endonuclease *Pst*I by amplification with mutagen primers, when the amplified fragment contains an A at position 6936, which corresponds to haplotype A1 or A2, so that when the amplified fragment contains a G, which corresponds to haplotype A3, it remains undigested.

15 **Kits**

According to an aspect of the invention, the A3 haplotype is detected by contacting the DNA of the subject with a nucleic acid probe, that is optionally labeled.

Primers may also be useful to amplify or sequence the portion of the
20 EPCR gene containing the polymorphic positions of interest.

Such probes or primers are nucleic acids that are capable of specifically hybridizing with a portion of the EPCR gene sequence containing the polymorphic positions of interest. That means that they are sequences that hybridize with the nucleic acid sequence to which it refers under conditions of
25 high stringency (Sambrook et al, 1989). These conditions are determined from the melting temperature T_m and the high ionic strength. Preferably, the most advantageous sequences are those which hybridize in the temperature range ($T_m - 5^\circ\text{C}$) to ($T_m - 30^\circ\text{C}$), and more preferably ($T_m - 5^\circ\text{C}$) to ($T_m - 10^\circ\text{C}$). A ionic strength of 6xSSC is more preferred. For instance, high stringency
30 hybridization conditions correspond to the highest T_m , e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases

are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (see Sambrook et al., supra, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., supra, 11.7-11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides ; preferably at least about 15 nucleotides ; and more preferably the length is at least about 20 nucleotides.

Preferred probes or primers are described in the legend to the figures or in the examples below.

When the primers are mutagenic primers, the above rules for determining the hybridisation conditions are to be adapted. The sequences of mutagenic primers may indeed partially vary from the wild-type sequence, so as to introduce restriction site when a given allele is amplified. Furthermore such mutagenic primers are frequently 30 to 40 nucleotide-long.

The present invention further provides kits suitable for determining at least one of the polymorphism of the A3 haplotype of the EPCR gene.

The kits may include the following components :

- (i) a probe as above defined, usually made of DNA, and that may be pre-labelled. Alternatively, the probe may be unlabelled and the ingredients for labelling may be included in the kit in separate containers ; and
- (ii) hybridization reagents : the kit may also contain other suitably packaged reagents and materials needed for the particular hybridization protocol, including solid-phase matrices, if applicable, and standards.

In another embodiment, the kits may include :

(i) sequence determination or amplification primers : sequencing primers may be pre-labelled or may contain an affinity purification or attachment moiety ; and

(ii) sequence determination or amplification reagents : the kit may also contain other suitably packaged reagents and materials needed for the particular sequencing amplification protocol. In one preferred embodiment, the kit comprises a panel of sequencing or amplification primers, whose sequences correspond to sequences adjacent to at least one of the polymorphic positions, as well as a means for detecting the presence of each polymorphic sequence.

10 In a particular embodiment, it is provided a kit which comprises a pair of nucleotide primers specific for amplifying all or part of the EPCR gene comprising at least one of the positions of the SNPs that are identified herein, especially positions 1651, 3610 and 4216 of SEQ ID No:1 (or SEQ ID No: 2).

15

The below figures and examples illustrate the invention without limiting its scope.

20

LEGENDS TO THE FIGURES :

Figure 1 shows the location of the amplification and sequencing primers on the EPCR gene.

25 Exons are symbolized by boxes. The 5' part of exon 1 and the 3' part of exon 4, which are noncoding, are striped.

30 The primer pairs used to amplify nearly the entire gene in seven PCR runs are indicated, and the size of the amplification products (in base pairs) is indicated between brackets. The oligonucleotides are numbered according to the position of their 5' nucleotide on sequence AF 106202 (Genbank accession number, (SEQ ID No:1)), followed by Fr for sense primers or Rv for antisense primers. The sequences of the amplification primers are indicated below, from 5' to 3':

PCR1: 61Fr GCTGAAGTGGGCGGATCACC (SEQ ID No:3) and 1137Rv
TCTAGCCTGGGTCATGCGGC(SEQ ID No:4)

PCR2:1114Fr TCTTGCCGCATGACCCAGGC (SEQ ID No:5) and 2212Rv
GGAAGGAGGCCAGGAGATGG (SEQ ID No:6)

5 PCR3: 1511Fr CTCTTACTAAGGGTGACGCG (SEQ ID No:7) and 3540Rv
TCTGATGCCCCACGAGACAC (SEQ ID No:8)

PCR4: 2528Fr TCTCTACAGGGCAGGCAGAG (SEQ ID No:9) and 5012Rv
TCGTGTTGTTGGTGTCTGGG (SEQ ID No:10)

10 PCR5: 4640Fr AGGAGTGTCTCTTCCACTGC (SEQ ID No:11) and 5540Rv
CTTGATGAGAAGTGCGTGG (SEQ ID No:12)

PCR6: 4993Fr CCCAGACACCAACACCACGAT (SEQ ID No:13) and 7320Rv
GTCTGTCTTTGGAGGATGGG (SEQ ID No:14)

PCR7:7171Fr AGAGGTGGACAAAGTACTTGG (SEQ ID No:15) and 8158Rv
GGAAGCCAGCA TTTCCAGG (SEQ ID No:16)

15 Figure 2A shows the distribution of sEPCR levels in 100 healthy male
volunteers.

Figure 2B shows the concordance of sEPCR levels at two visits.

Figure 3 represents the three EPCR gene haplotypes :

20 The 13 polymorphisms found to be in complete linkage disequilibrium
defined three haplotypes designated A1, A2 and A3. The nucleotides are
numbered according to the Genbank sequence (accession number AF106202,
SEQ ID No:1).

Figure 4 represents the plasma sEPCR levels according to the genotype
in 100 healthy male volunteers.

25 The mean \pm S.D. (n, range ; median) were 79.4 \pm 16.6 ng/mL (20, 56.4-
108.2; 79.5), 84.6 \pm 16.3 (48, 50.5-126; 82.9), 314 \pm 218.7 (8, 211.3-854.4;235.1),
85.5 \pm 20.3 (18, 63.3-137.5; 77.8) and 196.7 \pm 46.2 ng/mL (6, 138.4-266.55;
190.5) for A1 A1, A1 A2, A1 A3, A2 A2 and A2 A3 subjects, respectively. When
excluding the subject with atypical high sEPCR levels (854 ng/mL), mean \pm

S.D. (n, range ; median) of A1A3 subjects were 237.9±20.2 ng/mL (7, 211.3-274.9; 233.05).

Figures 5A and 5B represent the rapid A3 haplotype identification method.

Figure 5A : Schematic representation of the part of the human EPCR gene exon 4 containing G 6936, which identifies the A3 haplotype. The 6936 mutagen primer contains two foreign nucleotides at positions n-4 and n-3 from the 3' end (indicated by asterisks) in order to create a restriction site for the endonuclease *Pst* I when the amplified fragment contains an A at position 6936, which corresponds to haplotype A1 or A2; the amplified fragment containing a G, which corresponds to haplotype A3, remains undigested. After genomic amplification using this primer and the 7190Rv primer, the PCR-amplified fragment contains a *Pst* I site (CTGCA/G; underlined) when nucleotide 6936 is an A. In the amplified fragment, the part corresponding to the primer is shown in lower letters.

Figure 5B : 2% agarose gel electrophoresis of digested PCR products obtained using 6936 mutagen and 7190Rv primers. Lane A to C: subjects homozygous for an A at position 6936 (A1/A1, A2/A2 and A1/A2, respectively); a restriction site for *Pst* I was created, allowing the amplified fragment to be completely digested into two fragments of 254 and 36 bp (the latter is not visible on the gel). Lane D: subject homozygous for a G at position 6936 (A3/A3): no *Pst* I restriction site is available, and the fragment remains undigested at 290 bp. Lanes E and F: subjects heterozygous A/G at position 6936 (A1/A3 or A2/A3, i.e. A3 "heterozygotes"): both patterns are visible, corresponding to the undigested (290 bp) and digested (254 bp) amplified fragments. Lane G: Undigested PCR-amplified fragment.

Figure 6A and 6B represent the plasma sEPCR levels according to the presence or absence of A3 alleles, in a series of 176 healthy female controls from the PATHROS case-control study.

Figure 6A : Distribution of sEPCR levels

Figure 6B : Correlation between the genotype and the sEPCR level.

EXAMPLE : Identification of a haplotype of the EPCR gene that is associated with increased plasma levels of sEPCR and is a candidate risk factor for thrombosis

Subjects, Materials and Methods

- Materials

Evacuated tubes were from Beckton Dickinson (Le Pont de Claix, France).
10 The Qiamp Maxi kit was from Qiagen (Courtaboeuf, France). The sEPCR Asserachrom kit was kindly supplied by Stago Laboratories (Asnières, France). The DNA sequencing kit (Big Dye Terminator V3.0 Cycle Sequencing Ready Reaction with AmpliTaq DNA Polymerase FS) and the ABI Prism 3700 sequencer were from Applied Biosystems (Applera, Courtaboeuf, France). The
15 dNTP mix was from Amersham Biosciences Europe (Orsay, France). Oligonucleotides were from Proligo (Paris, France). Plates for amplified-product purification were from Millipore (Saint-Quentin en Yvelines, France). *Pst* I restriction endonuclease was from New England Biolabs (Ozyme, Saint Quentin en Yvelines, France). Agarose was from Life Technologies (Invitrogen,
20 Cergy-Pontoise, France).

- Healthy subjects and patients

One hundred unrelated healthy Caucasian male volunteers aged from 18 to 35 years were recruited and studied at the Clinical Investigations Center of Hôpital Européen Georges Pompidou. This population has been described in
25 detail elsewhere (Fontana et al. (2003) ; Dupont et al. (2003)). Briefly, the volunteers were non smokers and had not taken any medication for at least 10 days before blood sampling. Volunteers with a personal or family history of excessive bleeding or thrombosis were excluded. The subjects underwent a physical examination and routine laboratory tests ((Fontana et al. (2003) ;
30 Dupont et al. (2003)), including C-reactive protein and F1+2 assay.

Blood was collected from all volunteers by venipuncture in tubes containing 0.11 M sodium citrate (1 vol/9 vol) on day 1 (visit 1) and day 7 (visit

2). Plasma was obtained by centrifugation at 2300 g for 20 min, and was immediately subjected to routine laboratory tests or stored at -80°C until use. Genomic DNA was isolated from peripheral blood mononuclear cells by using the Qiamp Maxi kit according to the manufacturer's instructions.

- 5 A group of 338 patients, matched for age and sex with 338 controls, were studied in a second phase. These subjects had participated in a case-control study, the PARIS THROMbosis Study (PATHROS), designed to seek genetic risk factors for venous thromboembolism (VTE). The inclusion and exclusion criteria applied to cases, and their clinical and biological characteristics, have been
- 10 extensively described elsewhere (Arnaud et al. (2000)). Briefly, the patients had had at least one episode of objectively diagnosed deep venous thrombosis (documented by compression and ventilation lung ultrasonography or venography) and/or pulmonary embolism (documented by perfusion and ventilation lung scanning, convention pulmonary angiography, or computed
- 15 tomographic angiography). The controls were healthy European subjects recruited from a healthcare center to which they had been referred for a routine checkup. Subjects with a history of VTE, arterial disease or known malignancy were excluded on the basis of a medical questionnaire. To avoid a possible
- 20 bias due to the fact that controls came from a health care center, the inventors checked that the frequencies of factor V and Prothrombin G20210A mutations were similar to that observed in other control populations (Arnaud et al., 2000 ; Emmerich et al., 2001).

The study protocols were approved by local ethics committee.

- 25 Blood was collected and plasma prepared as previously described (Arnaud et al. (2000)). DNA was extracted from white blood cells by using a standard method (Miller et al. (1998)). Factor V Arg506Gln and prothrombin gene G20210A mutations were identified as previously described (Alhenc-Gelas et al. (1999)). All samples were obtained with the participants' informed consent.

- Soluble EPCR assay

Soluble EPCR (sEPCR) levels were determined in plasma by using sEPCR Asserachrom ELISA kits from the same batch, according to the manufacturer's instructions.

5 - EPCR gene screening for polymorphisms

The nucleotides of the EPCR gene were numbered according to the sequence available under GenBank accession number AF106202 (SEQ ID No:1). As shown in figure 1, seven amplification fragments spanned more than 99% of the EPCR gene. The location of the amplification primers relative to the
10 EPCR gene sequence, as well as their nucleotide sequences, are indicated in figure 1 and its legend. Each amplification product was purified by filtration on Millipore plates and sequenced with a DNA sequencing kit according to the manufacturer's instructions; the sequencing products were analyzed on ABI Prism 3700 sequencer.

15 Screening of 40 subjects from a given population is sufficient to identify polymorphisms having a frequency of 5% or more, with a confidence interval (CI) of 95%. Thus, the entire EPCR gene of the first 48 consecutive healthy volunteers was screened for polymorphisms as described above. Then, the polymorphic sites identified in these 48 subjects were screened for in 52
20 additional healthy subjects, with primers targeting these sites.

- Haplotype A3 identification in the PATHROS population.

A rapid method of A3 haplotype identification was developed by using the G at nt 6936 of the EPCR gene as marker. The region surrounding nucleotide 6936 was amplified by using a mutagenic 35-mer 6936 mutagen (5'-
25 CCTACACTTCGCTGGTCTGGCGTCCTGGTctgc-3' (SEQ ID No:17) as upstream primer, and a 22-mer 7190Rv (5'-CAAGTACTTGTCCACCTCTCC-3' (SEQ ID No: 18) as downstream primer. The upstream primer bore two foreign nucleotides (underlined lowercase characters in the preceding sequence), thereby allowing amplified fragments bearing an A at position 6936 of the EPCR gene (thus
30 corresponding to haplotype A1 or A2) to be cleaved by the restriction endonuclease *Pst* I, whereas amplified fragments bearing a G (and

corresponding to haplotype A3) remained undigested. Twenty microliters of the 290-bp amplification product was incubated overnight at 37°C with 20 units of *Pst* I, and digestion was checked by electrophoresis on 2% agarose gel.

- Statistical analysis

5 Continuous variables are reported as means and standard deviation or as medians and range (according to their distribution), and categorical variables are reported as counts and percentages. Skewed variables were log-transformed before analysis. Individual subjects' s EPCR plasma concentrations at visits 1 and 2 were compared using a concordance test (Lin
10 (1989)). The chi-square test was used to compare the observed genotype frequencies with the Hardy-Weinberg equilibrium prediction. The association between the genotype and the biological phenotype (sEPCR level) was tested using ANOVA. Comparisons between case and control subjects were based on Student's unpaired t-test for continuous variables, and the chi-square test or
15 Fisher's exact test for categorical variables. Multivariate analysis was used to determine the odds ratio, based on multiple logistic regression. Statistical tests were run on Statview5® statistical software (SAS), and differences with *P* values <0.05 were considered statistically significant.

20

Results

To assess the intra-individual variability of plasma sEPCR levels, the inventors tested two blood samples, obtained one week apart, from each of 100 healthy male volunteers, except for two subjects who did not attend visit 2. At least two phenotypic groups were identified. Values in both groups had a
25 gaussian distribution (Figure 2A); sEPCR levels were below 137.5 ng/ml (3 nM) at both visits in 84 subjects, and above 138.5 ng/ml at both visits in 14 subjects. Plasma sEPCR levels concorded between the two visits ($R^2 = 0.95$, $P < 0.0001$)(Figure 2B). One subject had a very high sEPCR level (854 ng/mL) at both visits. As plasma sEPCR levels may be influenced by inflammation, CRP
30 levels were also determined in all the subjects. The results (CRP values always below 5 mg/mL) ruled out a role of inflammation in the bimodal distribution of sEPCR levels. F1+2 levels in the 84 subjects with lower sEPCR levels (median

1.03 nM; range 0.56-3.45) were similar to those in the 14 subjects with higher sEPCR levels (median 1.28; range 0.68-3.12) ($P=0.57$), ruling out elevated thrombin generation in the latter group.

The existence of different phenotypic groups of sEPCR expression, together with the stability of individual levels over time, pointed to genetic control of the sEPCR level. The inventors therefore analyzed the EPCR gene in 48 consecutive healthy volunteers, from nucleotide 80 to 8100 (*i.e.* 99% of the available AF106202 sequence (SEQ ID No:1), corresponding to ~2300 nucleotides upstream of the ATG codon, the exons, the introns and ~1500 nt downstream of the stop codon). The inventors found 16 single nucleotide polymorphisms (SNP) located throughout the gene. The first was a C to G transversion of nucleotide (nt) 1651, located within the promoter region. Six other SNPs were located in intron 1 and affected nt 3610 (T to C transition), nt 3787 (T to C transition), nt 3877 (A to G transition), nt 4216 (C, G or A), nt 4414 (T to C transition) and nt 4868 (C to T transition). Four SNPs were located in intron 2, and affected nt 5233 (A to G transition), nt 5760 (C to T transition), nt 6146 (G to A transition) and nt 6333 (C to T transition). Exon 4 contained two SNPs, with an A to G transition at nt 6936, changing Ser 219 to Gly (Simmonds *et al.* (1999)), and a C to G transversion at nt 7014, in the non coding part of exon 4. Finally, three SNPs were located in the 3' untranslated (3'-UTR) part of the gene; they consisted of a C to G transversion at nt 7966, a G to A transition at nt 7968 and an A to G transition at nt 7999. These polymorphisms had allelic frequencies above 0.05, except for 4414C and 6146A (0.041 and 0.036, respectively).

Using primers targeting the 14 frequent polymorphic positions, the inventors amplified and sequenced the corresponding regions of the EPCR gene in the other 52 healthy volunteers in order to determine the allelic frequencies of the polymorphisms. All but one (nt 7966) of the 14 frequent SNPs were in complete linkage disequilibrium. These 13 SNPs defined three haplotypes, that were designated A1, A2 and A3. As shown in figure 3, A1 and A2 were major haplotypes, with allelic frequencies of 0.48 and 0.45, respectively. The A1 haplotype consisted of a combination of T at nt 3787, A at

nt 3877, C at nt 4216, C at nt 4868, A at nt 5233, C at nt 5760, C at nt 6333, C at nt 7014, G at nt 7968 and A at nt 7999. The A2 haplotype was a combination of C at nt 3787, G at nt 3877, G at nt 4216, T at nt 4868, G at nt 5233, T at nt 5760, T at nt 6333, G at nt 7014, A at nt 7968 and G at nt 7999. A3, the least common haplotype (allelic frequency 0.07), differs from A2 haplotype at four nucleotide positions (G at nt 1651, C at nt 3610, A at nt 4216 and G at nt 6936). The allelic frequencies of the three haplotypes were in Hardy-Weinberg equilibrium (χ^2 test, $p>0.05$).

To establish whether or not the plasma sEPCR level is genetically regulated, the inventors compared the plasma sEPCR level with the sEPCR genotype (Figure 4). As plasma sEPCR levels were stable between the two visits, we used the mean value for each subject. As shown in figure 4, sEPCR levels were significantly higher in subjects carrying one A3 allele (A1 A3 or A2 A3) than in subjects carrying no A3 allele. No significant difference in sEPCR levels was observed between A1 A1 or A2 A2 homozygotes and A1 A2 heterozygotes. The mean sEPCR level in subjects having at least one A3 allele was 264 ± 174 ng/ml (range 138.5 to 854) [218.9 ± 39.36 ng/ml (range: 138.5 to 274.9) after excluding the subject with a value of 854 ng/mL], compared to 83.6 ± 17.2 ng/ml (range: 50.5 to 137.5) in the other subjects ($p<0.0001$, 95% CI). It is important to underline that all the subjects carrying the A3 haplotype had elevated sEPCR levels at both visits, one week apart. Interestingly, none of the 100 volunteers was an A3 A3 homozygote.

To evaluate the possible influence of sEPCR levels on the risk of venous thromboembolism, the inventors investigated a cohort of 338 patients matched for age and sex with 338 healthy controls from the PATHROS study. The patients were 162 men (47.9%) and 176 women (52.1%). Mean age was 46 ± 13 years in the control group and 48 ± 15 years in the patient group ($P=0.06$). The main characteristics and clinical events of the patients and controls are shown in Table 1 :

Table 1. Characteristics of the PATHROS case-control study population

	Cases (n=338)	Controls (n=338)	P
5 Sex ratio (men/women)	162/178	162/178	
Age (years)	48 ± 15	46 ± 13	0.06
OC or HRT in females, %	28.9	40.2	
Pulmonary embolism, %	43.5	
Recurrent thrombosis, %	33.2	
10 Primary thrombosis, *%	25.0	
Factor V Arg506Gln, %	18.6	4.2	<0.0001
Factor II G20210A Mutation %	11.9	4.5	0.0003

15 *Excluding acquired risk factors: pregnancy, cancer, surgery, and immobilization
 OC= oral contraceptive HRT= hormonal replacement treatment

20 The factor V Arg506Gln mutation was detected in 18.6% of cases and 4.2% of controls ($P < 0.0001$), and the prothrombin G20210A mutation in 11.9% of cases and 4.3% of controls ($P = 0.0003$). These frequencies are similar to those observed in other European populations.

25 To identify subjects bearing an A3 allele, the inventors developed a rapid screening method for this haplotype (for details see Figure 5). Using this method, the inventors identified 89 patients (26.3%) carrying at least one A3 allele (4 were homozygous and 85 "heterozygous"); 60 controls (17.7%) carried an A3 allele (2 were homozygous and 58 "heterozygous") ($P = 0.009$) (Table 2).

Table 2: Repartition of A3 haplotype in cases and controls in the PATROS study, in the whole population or according to gender, and associated risk for venous thrombosis

	Total		Men		Women	
	Case	Controls	Case	Controls	Case	Controls
n	338	338	162	162	176	176
age mean \pm sd (years)	48 \pm 15	46 \pm 13	52 \pm 14	49 \pm 12	45 \pm 14	44 \pm 13
Number of subjects with :						
no A3 allele	249	278	116	136	133	142
one A3 allele	85	58	45	26	40	32
two A3 alleles	4	2	1	0	3	2
A3 carriers (%)	26.3	17.7	28.4	16	24.4	19.3
	$P = 0.009$		$P = 0.01$		$P = 0.3$	
Allele frequency :						
A3	0.138	0.092	0.145	0.08	0.13	0.1
A1 + A2	0.862	0.908	0.855	0.992	0.87	0.9
OR						
Univariate analysis [CI 95%]	1.7 ($P=0.007$) [1.2-2.40]		2.1 ($P=0.008$) [1.2-3.6]		1.4 ($P=0.25$) [0.8-2.3]	
OR						
Multivariate analysis* [CI 95%]	1.8 ($P=0.004$) [1.2-2.6]		2.5 ($P=0.0017$) [1.4-4.5]		1.3 ($P=0.37$) [0.8-2.2]	
* multivariate analysis for sex, age, Factor V Leiden mutation and Prothrombin 20210A mutation						

* multivariate analysis for sex, age, Factor V Leiden mutation and Prothrombin 20210A mutation

The allelic frequency of the A3 allele was 0.092 in the control subjects and 0.138 in the patients.

Surprisingly, the A3 haplotype distribution was gender-related, prompting the inventors to analyze men and women separately (Table 2). The A3 haplotype distribution remained significantly different ($P=0.011$) between male cases (28.4% were A3 carriers; allelic frequency 0.145) and male controls (16% were A3 carriers; allelic frequency 0.08). In contrast, there was no significant difference ($P=0.3$) between female cases (24.4% of A3 carriers; allelic frequency 0.13) and female controls (19.3% of A3 carriers; allelic frequency 0.1).

This latter finding led the inventors to examine whether the A3 haplotype was also associated with higher plasma sEPCR levels in women. They therefore assayed sEPCR in plasma from the 176 female controls. Like in the 100 healthy male volunteers population, the healthy female controls from the PATHROS study who carried the A3 haplotype had elevated sEPCR levels. In addition, sEPCR levels correlated with the number of A3 alleles: values were 77.5 ± 20.67 ng/ml (mean \pm 1 SD) in women with no A3 alleles ($n=142$), 211.1 ± 48.9 ng/ml in women with one A3 allele ($n=32$), and 483.2 ± 47.4 ng/ml in the two women who were homozygous for the A3 haplotype ($P<0.0001$). Thus, as in healthy men, the A3 haplotype is associated with elevated sEPCR levels in healthy women.

However, the frequency of hormonal treatment (oral contraception or replacement therapy) was higher in the female controls than in the female cases. This hinders the interpretation of the above results in the absence of data on the possible interaction of the A3 allele with hormonal treatment.

The inventors expect that the A3 allele is associated with a thrombosis risk in women without hormonal treatment, as it is in men.

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